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**14. ABSTRACT:** The aims of this project are to determine the phenotype and antileukemic activity of activated bone marrow infiltrating leukemia (MIL) and compare them to activated peripheral blood lymphocytes from patients with chronic myelogenous leukemia (CML) on imatinib or other tyrosine kinase inhibitor therapy. Bone marrow and peripheral blood specimens were obtained from CML patients who had at least a minor cytogenetic response. The phenotype of MILs and peripheral blood lymphocytes (PBL) was analyzed by flow cytometry. MILs and PBLs were expanded and activated with anti CD3/CD28 magnetic beads in culture for 10 days. Activated MILs and PBLs were characterized by flow cytometry and tested for antileukemic activity in a colony suppression assay by coculture with CD34+ bone marrow progenitors at varying CD3:CD34 ratios in methycullulose medium. Analysis of the phenotype of MILs from four patients showed that MILs are predominantly comprised of effector memory T cells. These MILs could be expanded effectively without change in phenotype. MILs as well as activated PBLs showed ability to suppress CML progenitor growth in vitro.

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Chronic myeloid leukemia, immunotherapy, marrow-infiltrating lymphocytes, phenotype, T-cell expansion

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DOD progress report-W81XWH-06-01-0277 (Pullarkat)

## Introduction

The leukemic cells in CML express various tumor antigens that are recognized by the immune system. Imatinib and other tyrosine kinase inhibitors, although highly effective are not curative therapy and patients invariably relapse upon discontinuation of these agents. Generation of an effective immune response has the potential to eradicate leukemia and achieve cure. Relapse of CML can occur after allogeneic HCT, necessitating the use of donor lymphocyte infusions with its attendant toxicity, especially induction of severe GVHD. Thus better methods of augmenting allogeneic and autologous immune response against CML are needed.

Tumor-infiltrating lymphocytes (TILs) in solid tumors are comprised of activated T-cells that have specificity for tumor-associated antigens. Marrow infiltrating lymphocytes (MILs) are T cells analogous to TILs and have been isolated from the bone marrow of patients with some solid tumors like breast cancer. The MILs have been shown to be comprised of activated central and effector memory T-cells with a restricted T cell receptor repertoire suggesting that these cells represent a specific immune response to the malignancy. This study characterizes the phenotype and clonality of these MILs obtained from patients who have at least a minor cytogenetic response to tyrosine kinase inhibitor therapy. The ability to expand these MILs without change in their phenotype as well as the ability of these expanded and activated MILs to suppress leukemic cell proliferation is also being examined.

## Body

### *Phenotyping of MILs*

MIL and PBL obtained simultaneously were phenotyped in 4 patients so far. Analysis was performed on lymphocyte population gated by $CD45^{high}$ and low side scatter. CD4 and CD8 cells were classified as naïve, central memory or effector memory subtypes based on their expression of CD45RA and CCR7. Naïve cells were defined as CD45RA+, CCR7+ and central and effector memory cells were CD45RA-, CCR7+ and CD45RA-, CCR7- respectively. The percentages of various lymphocyte subsets are shown in Table 1. The predominant population was of effector memory phenotype (Fig. 1 and Table 1). Increased expression of the activation marker CD69 was seen after bead activation. Expression of the bone marrow homing receptor CXCR4 was maintained. Thus effector memory cells comprise most of the MILs.

### *Expansion*

MILs and PBLs were expanded and activated with anti CD3/CD28 magnetic beads in culture for 10 days in AIM-V medium with IL-2 at concentration of 50U/mL. Magnetic beads to T-cell ratio used was 1:1. The fold expansion achieved is shown in Figure 2.

The expansion achieved was lower than expected and we are currently optimizing this by altering the magnetic bead to T cell ratios. We are also examining the effect of varying T cell: bead ratios to obtain preferential expansion of CD8 cells. In the patients studied so far, expansion did not alter the phenotype of MILs or PBLs. (Figure 1)

*Colony suppression assays*

Colony suppression assay was performed in 3 patients and the percentage inhibition of colony growth was calculated. Activated MILs and PBL were capable of suppressing colony formation in a dose- dependent manner. In 3 patients tested, the percentages of growth inhibition at CD3: CD34 ratio of 100 were 68%, 96% and 42% for MILs. Corresponding values for PBL were 75%, 97% and 44%. Results from 2 patients is shown in Figure 3A and 3B. In these patients, MILs were equivalent to PBLs in inhibiting growth of BCR-ABL + colonies. We are currently examining the ability of CD8 cells isolated from expanded MILs and PBLs to kill CD34+ CML targets. Cytotoxicity is being examined by expression of the degranulation marker CD107 on the cell surface. We will also examine the ability of CD8 MILs and PBLs to suppress colony formation.

Key accomplishments

- Detailed characterization of immunophenotype of MILs
- Effective expansion of MILs without alteration of immunophenotype
- Demonstration of antileukemic activity of expanded MILs and PBLs by colony suppression assay.

Reportable outcomes

Results will be submitted to American Society of Hematology Annual Meeting December 2007 and a manuscript will be prepared after complete analysis of the first 5 samples is performed.

Conclusion

In the patients analyzed so far we have shown that memory CD4 and CD8 cells comprise the majority of MILs. Specifically these cells are of the effector memory phenotype. MILs and PBLs can be effectively expanded using CD3/CD28 beads. The expanded and activated MILs as well as PBLs show ability to suppress colony formation by CML progenitors. We did not detect any significant difference between activated MILs and PBLs with regard to colony suppression. We are currently optimizing the

expansion protocol by adjusting the T cell to bead ratio as well as using other approaches including blocking of PD-1 signalling. We are also testing expanded and activated CD8 cells (isolated from MILs and PBLs) for cytotoxicity by coculturing them with CD34+ CML progenitors, followed by flow cytometric analysis of CD107 expression by the coculturedT cells. CD107 is a marker for of degranulation and has been shown to reliably demonstrate cytotoxicity. T cell receptor spectratyping studies to assess clonality of MILs and PBIs are currently being performed. We are also analyzing additional patient samples. Due to the recent decline in use of allogeneic stem cell transplant for treatment of CML, we have not been able to analyze allogeneic MILs as originally proposed. Allogeneic MILS will be analyzed if such samples are available during the remainder of this project.

Supporting data

**Table 1. Immunophenotype of bone marrow and peripheral blood lymphocytes before and after expansion. Numbers denote percentages and are the mean from 4 patients**

| | BM | | PB | |
|---|---|---|---|---|
| | PRE | POST | PRE | POST |
| CD3 | 66.12 ± 9.59 | 86.66 ± 15.76 | 69.92 ± 8.62 | 87.65 ± 9.65 |
| CD8B | 21.81 ± 10.18 | 26.66 ± 15.60 | 19.93 ± 7.52 | 40.55 ± 22.92 |
| CD4 | 33.99 ± 8.89 | 49.86 ± 21.51 | 39.43 ± 7.32 | 36.80 ± 22.52 |
| CD56 | 19.15 ± 5.77 | 3.09 ± 2.48 | 12.94 ±7.23 | 8.31 ± 3.692 |
| CD19 | 8.74 ± 5.87 | 31.7± 23.23 | 9.66 ± 5.78 | 21.25 ± 35.43 |
| CD4+CD25+ | 2.49% ± 0.0243 | | 3.23% ± 0.0272 | |
| CD4 RA-CCR7- | 99.86% ± 0.0014 | 99.93% ± 0.0004 | 88.89% ± 0.2214 | 99.93% ± 0.000589 |
| CD8 RA-CCR7- | 83.03% ± 0.3356 | 99.79% ± 0.0031 | 81.58% ± 0.3607 | 99.95% ± 0.000574 |

**Figure 1. Imunophenotyping of MILs show that the predominant population is of effector memory subtype (CD45RA-, CCR7-) This phenotype is maintained after expansion**

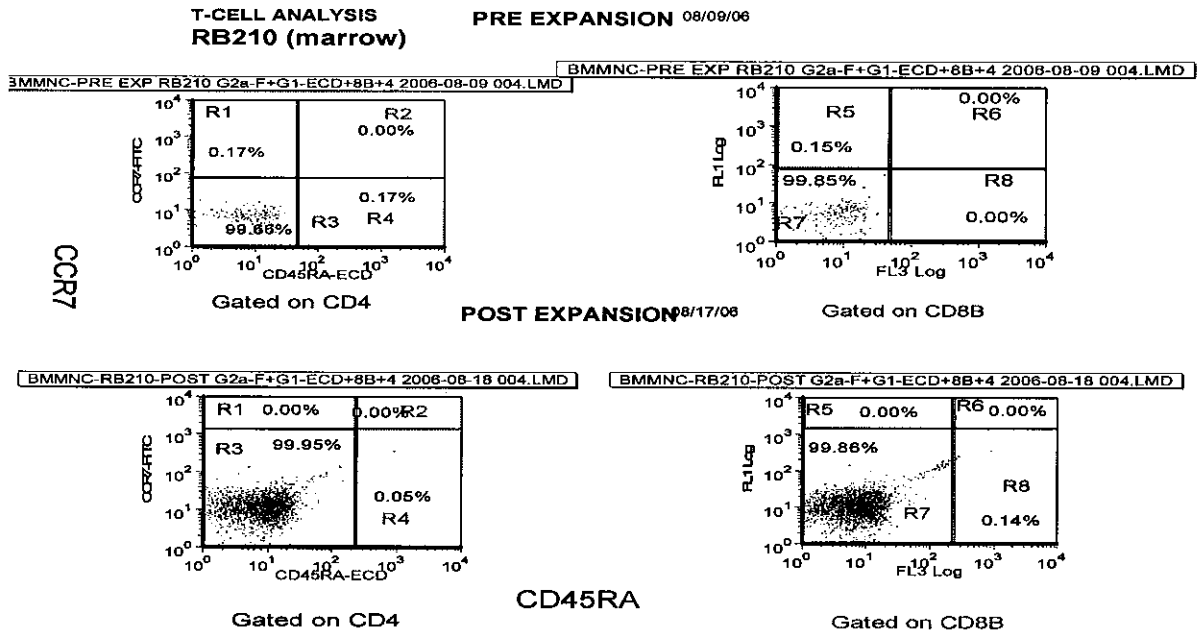


**Figure 2. Fold expansion of MILs and PBLs from 5 patients**

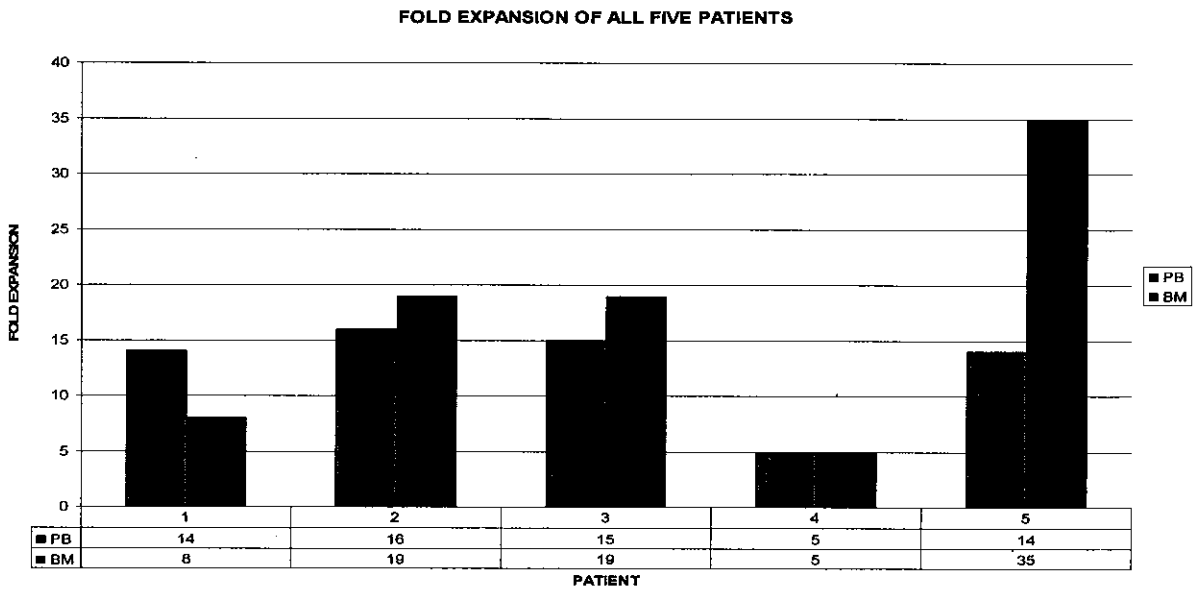

**Figure 3 A and 3B. Colony suppression assays from 2 representative patients show dose dependent supression ofCD34 progenitor frowth by expanded MILs and PBLs. % growth inhibition was calculated by the formula: % growth inhibition= [1-(colony # for that ratio ÷ colony # for CD34)] X 100**

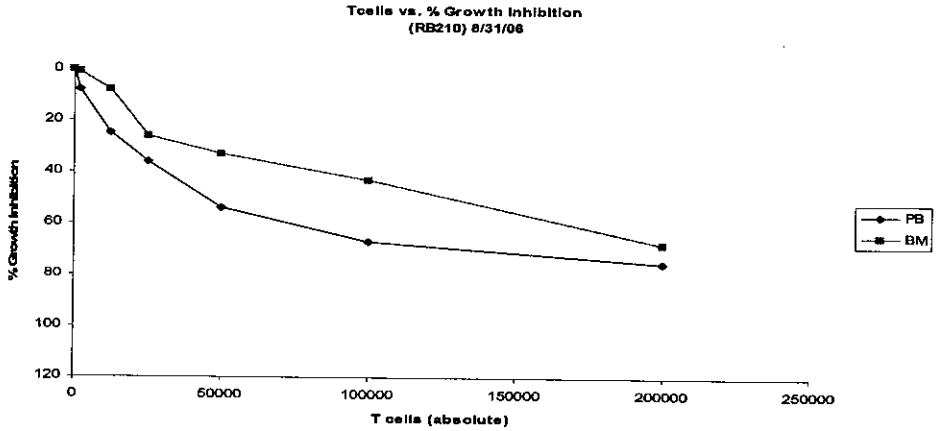


Figure 3A

Colony suppression assay

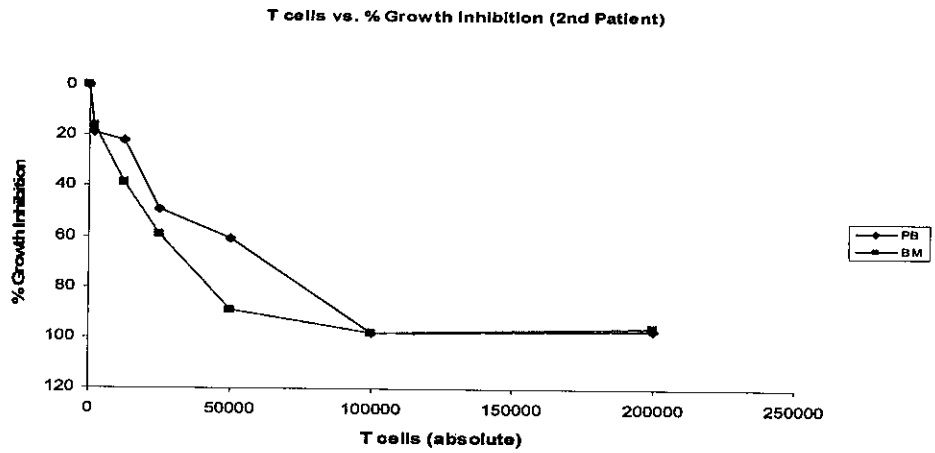


Figure 3B